Z. klin. Chem. u. klin. Biochem.
9. Jg., S. 415—418, September 1971

Rapid Determination of Urinary Total Porphyrins by Ion Exchange Chromatography

By M. Doss and A. Schmidt

Clinical Biochemistry Division, Hygiene Institute of the Philipps University Marburg/Lahn

(Eingegangen am 18. Juni 1971)

Total urinary porphyrins were eluted from a commercially available anion exchange chromatographic column with hydrochloric acid and determined spectrophotometrically. For 1 ml samples the optimum concentration range for measurement was found to lie between 0.1 and 1.5 mg of total porphyrin/l urine. Assay of a single sample takes 12 min. The specificity (measurement at three wave lengths), sensitivity (20 µg/l), and precision (variability coefficient 8%) are sufficient to provide rapid information about total porphyrins.
Normal values were determined from findings on healthy test persons: 55 ± 33 µg/l ($\bar{x} \pm s$). For 24 patients with secondary porphyrinuria and hepatic porphyrias, assay of total prophyrins by ion exchange chromatography was compared to analysis using thin-layer chromatography; similar results were usually obtained with both methods, but concentration differences of up to 30% were also found.

Die Gesamtporphyrine des Urins wurden aus einer kommerziell verfügbaren Anionenaustauschchromatographiesäule mit Salzsäure eluiert und spektrophotometrisch bestimmt. Bei einem Probenvolumen von 1 ml ergab sich ein optimaler Meßbereich für Konzentrationen von 0,1 bis 1,5 mg Gesamtporphyrine/l Urin. Die Analysenzeit beträgt 12 Min. für eine Probe. Spezifität (Messung bei drei Wellenlängen), Empfindlichkeit (20 µg/l) und Präzision (Variabilitätskoeffizient 8%) sind für eine schnelle Information über Gesamtporphyrine ausreichend.
Normalwerte wurden in einer Stichprobe von 95 gesunden Probanden bestimmt: 55 ± 33 µg/l ($\bar{x} \pm s$). Bei 24 Patienten mit sekundären Porphyrinurien und hepatischen Porphyrien wurden die Gesamtporphyrine nach Ionenaustauschchromatographie mit dünnschichtchromatographischen Bestimmungen verglichen; neben Übereinstimmungen der Resultate fanden sich Konzentrationsdifferenzen bis zu 30%.

Determination of total urinary porphyrins as copper chelates has proved in practice to be too time consuming for use in the clinical chemical routine laboratory (1). Although modifications of this method involving conversion of urinary porphyrins into zinc chelates (2) and centrifugation of the talcum adsorbate or assay of porphyrin methyl esters after rapid esterification (3) in methanol-sulfuric acid were considerable improvements (4), we prefer for the estimation of total porphyrins the procedure described as a screening test by Castrow and coworkers (5), which takes advantage of commercially available, ready-made columns. In principle the method involves rough separation of the porphyrin fraction by ion exchange chromatography: The urine is filtered directly over an anion exchange resin, onto which the porphyrins are adsorbed and then eluted with hydrochloric acid.
In view of the genuine lack of an adequate quick method for the assay of total porphyrins in urine, we decided to determine the feasibility of rapid quantitative analysis of total urinary porphyrins, employing disposable ion exchange chromatographic resins. Our purpose was to develop the method so that it would be particularly well suited for clearly detecting the slight to moderate increases in porphyrin excretion such as are found in clinically occult disturbances of porphyrin synthesis.

Procedures

Subjects

A series of clinically healthy test persons consisting of the staff of the Hygiene Institute and students of the University in Marburg was studied. Each age group (16—25, 26—35, 36 — . . . 65 years) contained at least 14 persons. In addition, patients with secondary porphyrinuria in fatty liver and lead intoxication were examined, as well as patients with hepatic porphyrias such as acute intermittent porphyria, hereditary coproporphyria, chronic hepatic porphyria without clinical symptoms, and porphyria cutanea tarda.

Collection of urine samples

The studies were based on urine samples collected at random during the day and shielded from light. The pH was adjusted to 5—7, and the urine stored at —20° until analyzed.

Reagents

Disposable ion exchange columns were obtained from Bio-Rad Laboratories ("Porphyrin Detection Columns" with wash solution 3N HCl, Cat. No. 94002), Munich. The column is charged with the anion exchange resin AG 1—X 8, 50—100 mesh (Cl^-). They were stored at about 4°.
Hydrochloric acid 3N.

Equipment and instruments

Test tubes 16×160 mm (Fiolax No. 2771, Schott No. 2611021).
Recording spectrophotometer.

Reference compounds

Uro- and coproporphyrin (III and I) from the urine of patients with porphyria cutanea tarda were isolated as methyl esters in a highly purified form by thin-layer chromatography (3). Samples

of about 1—5 μg were hydrolyzed with 25% (w/v) HCl for 48 hours at room temperature, then dried in a vacuum over NaOH. The same procedure was used to convert coproporphyrin I methyl ester (Calbiochem) to its free acid form.

Ion exchange chromatography

Place the column on a test tube and wash the resin in the lower part of the column with about 8 ml of distilled water (≙ full reservoir); all the distilled water should be allowed to run through. Pipette 1 ml of urine along the wall of the column directly above the resin and elute interfering material with 8 ml of distilled water. Permit liquid to pass through, then place column on a second test tube. Elute porphyrins twice with two ml 3N HCl each time, allowing complete passage of the first two ml before starting with the second and final portion.

Absorption spectrophotometry

The Soret (S) maximum of the porphyrins was measured. Absorption by a 4 ml sample of a 3N HCl solution containing the porphyrins is registered in a double-beam spectrophotometer between 430 and 380 nm, path length 2 cm, using distilled water as the reference solution (Fig. 1).

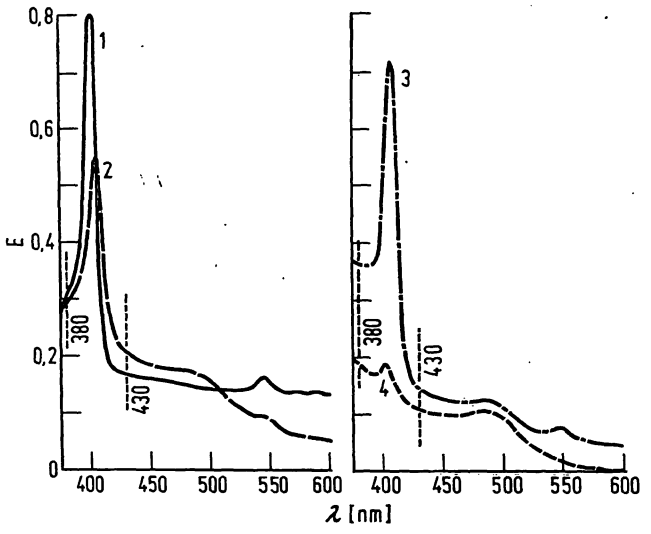

Fig. 1
Spectra of total urinary porphyrins (TUP) after elution from anion exchange resin 1 X-8 with 3N hydrochloric acid ("porphyrin column test"): 1 Reference compound coproporphyrin III in 3N HCl (1 mg/l), 2 TUP in case 23: chronic hepatic porphyria (483 μg/l), 3 TUP in case 15: acute intermittent porphyria (614 μg/l), and 4 TUP from a control person (56 μg/l). Sample volume 2 ml of urine. Elution volume 4 ml of HCl. Path length of the cuvettes 2 cm. Spectrophotometer Beckman DB-G

Calculations

$$E_{S\ corr.} = \frac{2\,E_S - (E_{430} + E_{380})}{k} \qquad (I)$$

$$\text{Correctional factor } k = 1.8 \qquad (II)$$

$$\text{Total porphyrins } (\mu g/ml) = E^{2\,cm}_{S\ corr.} \cdot 2.6 \qquad (III)$$

Derivation of formula III:
Millimolar extinction coefficients of uro- and coproporphyrin in 3N HCl:

uroporphyrin 547;

coproporphyrin 486.

The factor k used in correcting the extinction values for the free porphyrin acids in samples of biological origin (cf. 6) was determined on the basis of the absorption of the reference compounds in the Soret band:

$$k = \frac{2\,E_S - (E_{430} + E_{380})}{E_s} \qquad (IV)$$

$$k_{uroporphyrin} = 1.78 \qquad (V)$$

$$k_{coproporphyrin} = 1.80 \qquad (VI)$$

By transposing IV we obtain $E_{S\ corr.}$ as in I.
Taking the usual ratio of uro- to coproporphyrin in normal urine to be about 4:1 (7), a mean molecular weight of 691 and a mean millimolar extinction coefficient of 498 were determined for uro- plus coproporphyrin. These values are used in the following calculation of total porphyrins:
Total porphyrins (μg/ml urine) =

$$\frac{E_{S\ corr.} \cdot 691 \cdot \text{HCl volume (ml)}}{498 \cdot d\ (cm) \cdot \text{urine sample volume (ml)}} \qquad (VII)$$

If d = 2 cm, we arrive at the simplified formula III from equation VII.

Sensitivity

The amount corresponding to the doubled standard deviation of the individual determinations (2s) is about 20 μg/l. However, practice has shown that amounts as small as 5 μg/l can be distinguished from zero in 4 ml urine samples.

Accuracy

Of 10 μg of coproporphyrin added to 10 ml of normal urine, about 75% was recovered in each of eight separate samples.

Precision

Variability coefficients for two series (n = 8 each) were 6 and 12% ($\bar{x}$ = 126 μg/l).

Interfering factors

The extinction of the eluted porphyrins in 3N HCl decreased by about 25% in three hours in neon light at room temperature. In order to prevent such diminution, measurements should be made immediately following elution.
The corrected extinction value for normal urine with a low porphyrin content can be practically zero if the eluate contains substances whose absorption increases continuously between 440 and 370 nm and at 380 nm substantially exceeds that of the Soret band of the porphyrins.

Results

Total porphyrins in urine were assayed quantitatively with the aid of commercially prepared anion exchange columns. For 1 ml samples the optimum concentration range for measurement was found to lie between 0.1 and 1.5 mg of total porphyrin/l urine. A single analysis took 12 minutes. Typical spectra are shown in figure 1; the measure of concentration was the intensity of the absorption in the Soret region. The absorption maximum for the total porphyrin fraction in 3N hydrochloric acid after ion exchange chromatography was found as a rule between 402 and 404 nm. This absorption maximum is specific for porphyrins under the conditions prevailing here. Nevertheless, in urine containing large amounts of pigment other substances are also eluted from the resin AG 1—X 8 with hydrochloric acid. Since some emit a green fluorescence under UV light (366 nm), the Soret maximum cannot be relied upon as the sole parameter unless it is corrected (cf. 6).
Bilirubin in amounts up to 10 μg and hemin chloride in amounts up to 100 μg applied to the column could not be eluted under the conditions used for porphyrins. However, porphobilinogen in urine samples from patients with acute intermittent porphyria excreting up to 80 mg/l was detected with EHRLICH's reagent in

the hydrochloric acid porphyrin eluate. The color complex was spectrophotometrically identical to that of pure porphobilinogen. Porphobilinogen in the porphyrin eluate did not interfere with porphyrin absorption, because this monopyrrol exhibits no absorption between 450 and 360 nm. Therefore, the simultaneous elution of porphobilinogen does not restrict the specificity of the method for porphyrin estimation.

In 2% of the determinations of normal urine it was not possible to obtain a quantitative result, since the corrected extinction was zero due to relatively high background absorption by the eluate; nevertheless, a porphyrin absorption peak in the Soret region was clearly evident, and can be considered qualitative proof of the presence of porphyrin. The eluates of such samples were light pink to pale brown in color. Comparative studies (3) have shown that the porphyrin concentrations of these urine samples usually lie near the lower limit of normal.

Examination of a urine sample from a patient with porphyria cutanea tarda, which contained no interfering substances, revealed a linear relationship to the concentration for both the actually measured optical density at the Soret maximum and the corrected extinction, whereby the two values nearly coincided. A linear curve of the unadjusted extinctions in the Soret band corresponding to the actual concentrations was observed only for urine with an extremely high porphyrin content. For this reason the corrected extinction values were taken as the basis for calculation of the concentrations in unknown test samples. Correction is indispensible for determinations involving excretion of porphyrin to about 1 mg/l, as well as within the normal range.

Thin-layer chromatographic studies have revealed that normal urine contains less than 0.1 mg of porphyrin per liter (7). Since these concentrations lie within the lower range of the column method, 4 ml of urine was chromatographed for determination of the normal values. Under these conditions the optimum range of measurement was found to lie between 4 and 200 μg/l; the concentration curve was linear up to 500 μg/l. The normal values determined for the group of healthy test persons are given in table 1. They conformed to a normal distribution curve. On the basis of comparison with a urine sample which was included in each of the eight series as a standard (124 $\pm$ 14 μg/l, $\bar{x} \pm s$), a variability coefficient of 11% was found.

In table 2 data from the column assays are compared to results of thin-layer chromatographic analyses. The latter were obtained by taking the sum of the individual components after their separation in thin-layer chromatography and subsequent spectrophotometric measurement (3). In addition to the healthy persons, the study included patients with secondary coproporphyrinuria in fatty liver and chronic lead poisoning (7), with acute intermittent porphyria and hereditary coproporphyria in the latent phase (8), with chronic hepatic porphyria (8, 9), and with porphyria cutanea tarda (7—9). Considering the completely different principles of the

Table 1
Normal values of urinary total porphyrins in anion exchange chromatography

	n	Porphyrins (μg/l, $\bar{x} \pm s$)
Persons	95	55 ±33
♂	48	57 ±33
♀	47	47 ±33

Table 2
Comparison of results obtained by anion exchange chromatography (AEC-AS) and by thin-layer chromatography, both used in conjunction with absorption spectrophotometry (TLC-AS)

Groups studied	Subjects				Porphyrins (μg/l)	
	Number	Initials	Sex	Age	in AEC-AS	in TLC-AS
Healthy persons	1	E. S.	♀	20	47	49
	2	E. M.	♀	30	19	32
	3	R. V.	♂	21	107	31
	4	M. N.	♂	22	39	50
	5	S. Q.	♂	34	80	88
	6	G. S.	♂	35	39	30
	7	H. B.	♂	58	69	74
After alcohol consumption	8	A. B.	♂	22	180	181
	9	K. B.	♂	28	171	184
Alcoholic fatty liver	10	K. W.	♂	46	193	208
	11	H. F.	♂	42	278	254
Lead poisoning	12	G. L.	♂	31	668	565
Acute intermittent porphyria (in remission)	13	P. P.	♀	45	835	975
	14	H. B.	♂	49	525	755
	15	D. M.	♂	24	681	560
	16	A. B.	♀	33	222	153
Hereditary coproporphyria (in remission)	17	A. S.	♀	62	783	705
Chronic hepatic porphyria (without clinical symptoms)	18	W. E.	♂	58	130	166
Type A	19	A. H.	♂	52	350	349
	20	E. B.	♂	46	229	366
	21	W. W.	♂	47	243	367
Type B	22	A. G.	♂	57	123	183
	23	M. R.	♂	60	444	500
Type C	24	R. C.	♂	53	413	540
	25	P. L.	♂	55	692	613
Porphyria cutanea tarda	26	H. S.	♂	55	767	655
	27	G. L.	♂	52	861	915
	28	C. L.	♂	53	1320	1387
	29	J. D.	♀	50	2081	2458
	30	T. H.	♂	39	2544	3036
	31	M. D.	♀	53	5366	5500

two methods, the correlation is good in most cases. In all cases it was possible to distinguish unequivocally between normal and abnormal porphyrin excretion.
In ten samples from the normal group and in all samples with abnormally high porphyrin concentration the porphyrins measured by the column test were identified by comparing their behavior in thin-layer chromatography and their spectral properties to reference substances (3).

Discussion

To our knowledge, spectrophotometric assay of total porphyrins following anion exchange chromatography as described here and first developed as a qualitative porphyrin test by CASTROW and coworker (5) is the simplest and most rapid method for the quantitative detection of abnormal porphyrin excretion. The millimolar extinction coefficients of uro- and coproporphyrin in hydrochloric acid were found to be in agreement with previous measurements by other authors (cf. 6). Since the simple extinction of the Soret maximum for urine samples with normal or slightly to moderately elevated porphyrin content is not in agreement with the actual concentrations found in comparitive analyses, measurements at several wavelengths proved necessary, along with correctional factors (cf. 6). A yellow to brown coloration of the eluate can cause a relatively high background absorption, which is eliminated in calculation of the corrected extinction. The spectra were measured against distilled water (Fig. 1), since a blank value for a reagent would be too expensive for small series of tests; the use of corrected extinction values makes it superfluous anyway. A suggestion as to whether uro- or coproporphyrin predominates in a given urine sample can be obtained by exact registration of the absorption in the Soret region: A maximum at or above 404 nm indicates more uroporphyrin, whereas one at or below 402 nm indicates coproporphyrin.
If an elevated excretion of total porphyrins is apparent in column chromatography, further chromatographic and electrophoretic separation should be performed to determine which of the component porphyrins are elevated. Only then is one close to a specific diagnosis (10), which in the case of acute hepatic porphyrias or lead poisoning requires additional information on the excretion of porphyrin precursors (7). The porphyrin ion exchange test is well suited for rapid and sufficiently exact orientation concerning the severity of porphyrinuria in patients in whom a disturbance of porphyrin metabolism has been established on the basis of assays of the porphyrin precursors (7, 11) and analysis of the individual porphyrins (7—10). We use the test for definite and rapid exclusion of elevated porphyrin excretion and for follow-up control studies.

Urine of samples from patients were kindly provided
by Dr. H. HENNING and Dr. D. LOOK, Clinic Föhrenkamp of the BfA in Mölln (No. 18—23 and 25—31),
by Prof. Dr. G. STROHMEYER, Department of Medicine of the University Marburg (No. 10, 11, 13, 15, and 16),
by Dr. T. K. WITH, Svendborg County Hospital, Denmark (No. 17),
by Dr. K. H. SCHALLER, Institute for Work and Social Medicine of the University Erlangen-Nürnberg (No. 12 and 14),
and by Dr. A. LUCHMANN, Sanatorium of the LVA Rheinland-Pfalz for Liver and Metabolic Disorders (No. 24).

These studies were carried out with support of the Deutsche Forschungsgemeinschaft.

References

1. Doss, M. and W.-K. PHILIPP, this journal *7*, 148 (1969). — 2. Doss, M., Analytic. Biochem. (New York) *39*, 7 (1971). — 3. Doss, M., this journal *8*, 197 (1970). — 4. Doss, M., unpublished. 5. CASTROW II, F. F., J. F. MULLINS and G. C. MILLS, J. investigat. Dermat. *50*, 340 (1968). — 6. FALK, J. E., Porphyrins and Metalloporphyrins, Elsevier, Amsterdam (1964), page 171. — 7. Doss, M. and W. MEINHOF, Dtsch. med. Wschr. *96*, 1006 (1971). — 8. Doss, M., W. MEINHOF, D. LOOK, H. HENNING, P. NAWROCKI, W. DÖLLE, G. STROHMEYER and L. FILIPPINI, Proc. Intern. Conf. on Porphyrin Metabolism and the Porphyrias, Cape Town (1970), S. Afr. J. Laborat. Clin. Med., in press. — 9. Doss, M., D. LOOK, H. HENNING, C. J. LÜDERS, W. DÖLLE and G. STROHMEYER, this journal, in press. — 10. Doss, M., Klin. Wschr. *49*, 939, (1971). — 11. Doss, M. and A. SCHMIDT, this journal *9*, 99 (1971).

Professor Dr. Manfred Doss
Abteilung für Klinische Biochemie
Hygiene-Institut der Universität
D-355 Marburg a. d. Lahn
Pilgrimstein 2